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# The influence of Climate Change on the fate and behavior of different carbon nanotubes materials and implication to estuarine invertebrates

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ABSTRACT

The widespread use of Carbon nanotubes (CNTs) has been increasing exponentially, leading to a significant potential release into the environment. Nevertheless, the toxic effects of CNTs in natural aquatic systems are related to their ability to interact with abiotic compounds. Considering that salinity variations are one of the main challenges in the environment and thus may influence the behavior and toxicity of CNTs, a laboratory experiment was performed exposing the tube-building polychaete *Diopatra neapolitana* (Delle Chiaje 1841) for 28 days to pristine multi-walled carbon nanotube (MWCNTs) and carboxylated MWCNTs, maintained at control salinity 28 and low salinity 21. An innovative approach based on thermogravimetric analysis (TGA) was adopted for the first time to assess the presence of MWCNTs aggregates in the organisms. Both CNTs generated toxic impacts in terms of regenerative capacity, energy reserves and metabolic capacity as well as oxidative and neuro status, however greater toxic impacts were observed in polychaetes exposed to carboxylated MWCNTs. Moreover, both CNTs maintained under control salinity (28) generated higher toxic impacts in the polychaetes compared to individuals maintained under low salinity (21), indicating that exposed polychaetes tend to be more sensitive to the alteration induced by salinity variations on the chemical behavior of both MWCNTs in comparison to salt stress.

## 1. Introduction

Carbon nanotubes (CNTs) are artificial carbon-structured substances formed by one to more than one hundred graphene layers rolled into cylindrical tubes (Zhao and Liu, 2012). The current typical industrial CNTs types with one graphene layer are called single-walled carbon nanotubes (SWCNTs), while tubes containing more than one layer are multi-walled carbon nanotubes (MWCNTs) (Jackson et al., 2013). As a result of their unique chemical, physical and electronic properties, CNTs became potentially useful in a wide variety of applications, such as water treatment, medical applications, optics, electronic engineering, photovoltaic devices, automotive industry, sports equipment and cosmetics (Zhao and Liu, 2012; Freixa et al., 2018). In the last decade, the widespread use of CNTs increased exponentially leading to a significant potential release into the environment (Potočnik, 2011; Sanchez et al., 2012). In aquatic ecosystems, CNTs can be accumulated in river sediments, or remain suspended in the water column and transported to the marine systems (Freixa et al., 2018). The predicted environmental concentrations (PECs) of CNTs in aquatic environments have been estimated, by modeling studies, to range between 0.001 and 1000 μg/L (Zhang et al., 2017). Nevertheless, the environmental behavior and effects of CNTs in natural aquatic systems are related to their ability to interact with abiotic and biotic compounds and aggregate, creating clusters that exhibit a colloidal behavior (Khosravi-katuli et al., 2017). Generally, because CNTs are nearly insoluble in any solvent their application is limited (Mwangi et al., 2012). For this reason, scientists have explored different methods to increase the solubility of CNTs such as for both practical use and toxicological research. As example, it has been already demonstrated that CNTs surface areas containing carboxyl groups are widely used as active sites which improves the solubility and biocompatibility of the material in a water media and may, consequently, increase their toxicity (Scheibe et al., 2010). However, an increase in chemical toxicity of CNTs when combined with abiotic factors (for example salinity) has been also demonstrated (Peng

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et al., 2009; Pham et al., 2016). A study conducted by Peng et al. (2009) investigated the precipitation of oxidized CNTs in water by salts. The results showed that CNTs concentration decreased slightly with aging time, indicating that only 15% oxidized CNTs settled for 30 days while 85% was still suspended in a water column. The implication of these findings could be resumed in a high availability of the materials for organisms' uptake.

Under a climatic change scenario, the Intergovernmental Panel on Climate Change (IPCC) predicted that salinity of surface water can be determined by a combination of factors, including river flow, tidal surges, rainfall as well as the influence of sea-level rise and other climatic variables (IPCC, 2014). Considering that the salinity gradient is one of the main characteristics of estuarine ecosystems acting as both external ecological factor and physiological environmental driver for aquatic organisms (Telesh and Khlebovich, 2010; Cloern et al., 2017), we investigated the possible influence of salinity changes in biological responses of an estuarine model species, the polychaetes *Diopatra neapolitana* (Delle Chiaje 1841) exposed to different CNTs materials. Moreover, the influence of these environmental changes on the structure of CNTs and a consequent interaction with this polychaete species, was also detected. Currently, in the literature there is no information regarding CNTs behavior alteration and toxicity, caused by salinity variations and possible related responsiveness of the polychaete *D. neapolitana*. For this, the impacts induced by chronic exposure (28 days) to unfunctionalized MWCNTs (Nf-MWCNTs) and functionalized MWCNTs (f-MWCNTs) at different salinities were evaluated, by measuring alterations induced on polychaetes regenerative capacity as well as metabolic capacity, oxidative and neuro status. The presence and accumulation of MWCNTs in *D. neapolitana* was innovatively assessed by thermogravimetric analysis (TGA). TGA represents an analytical technique in which the mass of a substance is monitored as a function of temperature or time as the sample specimen is subjected to a controlled temperature program in a controlled atmosphere. By applying the derivative operation to the thermal curves obtained by TGA, it is possible to detect the degradation temperature of the material subjected to analysis. Indeed, TGA may represent a valuable tool for the quantitative determination of the composition of composite materials, proving that the degradation temperatures of single components are widely separated from each other (Renneckar et al., 2004; Zhang et al., 2015). To the best of our knowledge TGA has never been used for the detection of MWCNTs in living organisms, but it could potentially emerge as a successful technique for the identification and quantification of MWCNTs in biological matrices since their degradation temperatures generally lie in-between those of organic and inorganic materials (Zhang et al., 2015; Wang et al., 2008).

## 2. Material and methods

### 2.1. Model species

The tube-building polychaete *Diopatra neapolitana* was selected as model organism for this study as it plays important ecological roles by acting as a food source for other marine biota and increasing the physical complexity and biodiversity of habitats (Arias et al., 2016). Also, it has been demonstrated that *D. neapolitana* is a good sentinel species of metal contamination (Freitas et al., 2012) organic matter enrichment (Carregosa et al., 2014), pharmaceutical drugs (Freitas et al., 2015), salinity shifts and pH decrease (Pires et al., 2015) and NMs (De Marchi et al., 2017c, 2017d).

### 2.2. Experimental set up

Polychaete specimens were collected in the Mira channel, the southern shallow arm of the Ria de Aveiro lagoon (Portugal). In the laboratory, organisms were pushed out from their tubes, and placed in different aquaria (20 L each) for laboratory acclimation period (20 days). The aquaria were filled with a mixture of fine and medium sediment from the sampling area (see sediment details in De Marchi et al. (2018a)). Artificial seawater with the salinity 28 was used by the addition of artificial sea salt (Tropic Marin® Sea Salt) to deionized water, one day prior to utilization. Temperature was kept to 18 ± 1 °C, photoperiod of 12 h light: 12 h dark, pH 8.0 ± 1 and constant aeration. During this period every two-three days the specimens were fed *ad libitum* with small fragments of frozen cockles or mussels (Pires et al., 2012). To assess the impact of different CNTs on the regenerative capacity of *D. neapolitana*, immediately before the exposure assay individuals were removed from their new tubes, anaesthetized with a 4% $MgCl_2.6H_2O$ solution, and amputated at the 60th chaetiger under a stereomicroscope (Pires et al., 2012).

After the acclimation period, organisms were exposed for 28 days to two different salinities (21 and 28-control), each one combined with environmental relevant concentrations (0.001 and 0.01 mg/L) of two different CNTs (f-MWCNTs and Nf-MWCNTs). Before the beginning of the experiment, to reach salinity 21, the salinity was progressively (1–2 units) decreased to avoid additional osmotic stress to polychaetes.

The two salinity levels were chosen considering: i) the seasonal mean value of salinity for the sampling area (salinity 28) by considering levels identified in estuarine habitats (Santos et al., 2007; Portuguese Institute for Sea and Atmosphere, I. P. (IPMA, IP), n.d.); ii) extreme weather events such as the increases in fresh water runoff induced by global warming (IPCC, 2014), which caused negative salinity anomalies (i.e. a surface salinity that is less than salinity measured at depth of a few meters) (Asher et al., 2014) (salinity 21).

Regarding the CNTs, we selected two types of MWCNTs: one pristine (Nf-MWCNTs) and the other one chemically functionalized (f-MWCNTs) by introducing polar groups such as carboxyl groups (-COOH), both at the environmental relevant concentrations of 0.001 and 0.01 mg/L. The selection of these two CNTs was based on their different physical and chemical properties and different behavior on the water media (aggregation/disaggregation, adsorption/desorption, sedimentation/resuspension and dissolution) (Arndt et al., 2013). In particular the carboxylated CNTs are more stable in salt water media in comparison to pristine CNTs as a consequence of their oxidation process which introduces oxygen-containing groups on the CNTs surface. These groups ionize in water charging the oxygen atoms negatively and in aqueous phase the electrostatic repulsive forces between negative surface charges of the oxygen-containing groups can lead to stability of oxidized CNTs in the water column increasing the availability of these materials for the organisms (Peng et al., 2009). The selection of the CNTs was also based on their industrial applications. Three main properties of MWCNTs are specifically interesting for the industry: the electrical conductivity (as conductive as copper), their mechanical strength (up to 15 to 20 times stronger than steel and 5 times lighter) and their thermal conductivity (same as that of diamond and more than five times that of copper). The combination of these properties enables a whole new variety of useful applications (Li et al., 2011). In details, f-MWCNTs are used as additives in polymers, catalysts electron field emitters for cathode ray lighting elements, flat panel display, gas-discharge tubes in telecom networks, electromagnetic-wave absorption and shielding, energy conversion, lithium-battery anodes, nanotube composites, nanoprobes, nanolithography, nanoelectrodes, drug delivery sensors reinforcements in composites and supercapacitor (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com). The Nf-MWCNTs are used in different markets such as transportation (automotive, aeronautic, boats), electronics (electronic packaging, EMI-shielding, sensors), energy (Lithium-ion), industrial applications (Oil& Gas, dynamic rubber parts, coatings, heating elements) and sport goods (http://www.nanocyl.com/product/nc7000/). Moreover, for the choice of these nanoparticles, it was also considered the Organization for Economic Co-operation and Development (OECD)'s Working Party on Manufactured Nanomaterials (WPMN), which launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials

(OECD, 2010). This program promotes international co-operation on the human health and environmental safety of manufactured nanomaterials, and involves the safety testing and risk assessment of engineering nanoparticles. The OECD WPMN has published a list of ENPs selected considering their commercial use, production volume of the materials, availability of such materials for testing and the existing information that would readily be available on the materials and this list comprised the CNTs.

The exposure concentrations of both MWCNT were selected considering the PECs (0.001–1000 μg/L) of CNTs in aquatic systems (Zhang et al., 2017) and their toxic effects observed on different invertebrate species. For example, Mwangi et al. (2012) noticed a significantly reduction in survival and growth in mussels exposed to MWCNTs. Other studies have demonstrated that MWCNTs in bivalves can generated lysosomal damage (Miller et al., 2015), accumulation of these materials in intestinal lumen and digestive gland and histopathological changes in the epithelium and swelling of the connective tissue (Anisimova et al., 2015); oxidative stress and alteration of energy-related responses and metabolism (Andrade et al., 2018; Freitas et al., 2018; De Marchi et al., 2017a, 2017b, 2017d). Studies testing the impacts of CNTs in polychaetes are less, but also confirmed cellular damage and alteration in organisms energy reserves and metabolism (De Marchi et al., 2017c, 2018a, 2019).

For each condition, inside each aquarium, individuals used for the regenerative capacity analysis (n = 9 total) were separated from the ones used for the biochemical analysis (n = 9 total) using a plastic barrier in each aquarium.

### 2.3. CNTs in the exposure media and organisms

#### 2.3.1. Materials description

Both pristine and carboxylated MWCNTs were produced via the Catalytic Chemical Vapor Deposition (CCVD) process and characterized using Scanning Electron Microscopy (SEM) and Transmission electromicrographs (TEM). The Nf-MWCNTs were purchased from Nanocyl S.A. (MWCNTs: NC7000 series, http://www.nanocyl.com) (Diameter (nm): 9.5; Length (μm): 1.5; Carbon Purity (%): 90; Surface Area ($m^2$/g): 250–300) while f-MWCNTs from Times Nano: Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences (MWCNTs-COOH: TNMC1 series, http://www.timesnano.com) (Diameter (nm): 2–5; Length (μm): 10–30; Carbon Purity (%): 98; Surface Area ($m^2$/g): 400; Amorphous Carbon (mol%): 8–10; −COOH (wt%): 3.86).

Both CNTs were weighed (stock solution of 50 mg/L) and suspended in seawater (depending on the test condition, control-28 and low-21). Both materials were sonicated using a Hz ultrasound bath (IKA Labortechnik IKASONIC U50): 1 h for Nf-MWCNTs, while the f-MWCNTs, due to the presence of carboxyl groups (Shahnawaz et al., 2010), was sonicated for few minutes. Both Nf-MWCNT and f-MWCNT concentrations were re-established weekly after complete water renewals to ensure the same exposure concentrations during the experiment. The added MWCNTs (f and Nf) were maintained homogenously dispersed in the seawater using one submersible circulation pump *per* aquarium, increasing CNTs mass suspended in the water column (Vonk et al., 2009).

#### 2.3.2. Characterization analysis in the water media

The average size distribution measured by dynamic light scattering (DLS) and the polydispersity index (PDI) of both MWCNTs suspensions at different salinity levels were analyzed. As reported in the literature, DLS measurements represent a well-established method for the determination of the average diameter of CNTs in aqueous dispersions (Liu et al., 2011). In the present work, DLS measurements were carried out to obtain data regarding the tendency to aggregate and the settling behavior of suspended CNT materials in aqueous media. Measurements were performed on 1000 μL of suspension in four samples *per* condition, and five analyses *per* sample performed by DLS using a DelsaTM NanoC Particle Size Analyzer (Beckman Coulter). Each analysis was carried out by performing 120 acquisitions. Due to the inherent heterogeneity and colloidal instability of the analyzed samples, DLS analyses were repeated several times to ensure reproducible results. Intensity distributions were obtained by analyzing the autocorrelation functions through the Contin algorithm which is particularly appropriate for polydisperse and multimodal systems (Varenne et al., 2016). The cumulant method was used to obtain information on the particle's average hydrodynamic radii and on the PDI (Tardani and Mesa, 2015).

#### 2.3.3. Characterization and bioaccumulation in the organisms

Thermogravimetric Analysis (TGA) was performed on different entire tissue samples (10 mg) by using a TGA Q500 instrument (TA Instruments, Italy) in the temperature range 30–900 °C, at heating rate of 10 °C/min and under air flow of 60 mL/min. TGA, which records the weight loss of the materials as a function of temperature in a chosen atmosphere, was used for the first time as innovative method to detect the presence of MWCNT materials in *D. neapolitana* organisms exposed to aqueous media at different salinity levels containing different concentrations of the selected contaminants. A preliminary study was carried out to investigate the feasibility of using TGA by analyzing the organisms exposed to the maximum and minimum values of the concentration range of the MWCNT materials used (0.001 and 0.01 mg/L): (NF_S21: organisms exposed to Nf-MWCNTs in an aqueous medium with salinity 21; NF_S28: organisms exposed to Nf-MWCNTs in an aqueous medium with salinity 28; F_S21: organisms exposed to f-MWCNTs in an aqueous medium with salinity 21; F_S28: organisms exposed to f-MWCNTs in an aqueous medium with salinity 28) and non-contaminated organisms (CRTL S21_1, CRTL S21_2, CRTL S21_3: representing organisms immersed in an aqueous medium with salinity 21 and not exposed to carbon nanotubes; CRTL S28_1, CRTL S28_2, CRTL S28_3: organisms immersed in an aqueous medium with salinity 28 and not exposed to carbon nanotubes). The derivative of the TGA curves (DTG curves) were reported to better highlight the temperatures at which thermal degradation of the samples occurred, which could help to detect the presence of MWCNTs (f and Nf) by comparison with their DTG profile (Fig. 1).

### 2.4. Physiological parameter: regenerative capacity

Nine *D. neapolitana* specimens *per* condition (3 *per* aquarium) were analyzed every week during the experimental period (28 days). During the experiment, organisms for regenerative capacity analysis were inspected at day 11th, 18th, and 28th after amputation. The width of the regenerated body part was measured, and the number of new segments counted. Percentage of regenerated body width was calculated by comparing the width of the new segments with the width of the old segments (Pires et al., 2012). New segments were identified by the lighter colour and/or the narrower width compared to the old body segments (Pires et al., 2012).

### 2.5. Biochemical parameters: energy reserves and metabolic capacity, indicators of oxidative stress and neurotoxicity

At the end of the experimental period (28 days), the whole body of frozen organisms (3 *per* aquarium, 9 *per* condition) was individually pulverized with liquid nitrogen, divided in 0.2 g aliquots, and used for biochemical analyses. Extractions were performed with specific buffers to determine: a) energy reserves and metabolic capacity (protein (PROT) content, glycogen (GLY) content, electron transport system (ETS) activity); b) indicators of oxidative stress (lipid peroxidation (LPO) levels, reduced (GSH) and oxidized (GSSG) glutathione ratio, superoxide dismutase (SOD) activity; catalase (CAT) activity, Glutathione S-transferases (GSTs) activity); c) and neurotoxicity (Acetylcholinesterase (ATChI-ChE) activity). The methodologies used to perform each specific biomarker are described in detail in De Marchi

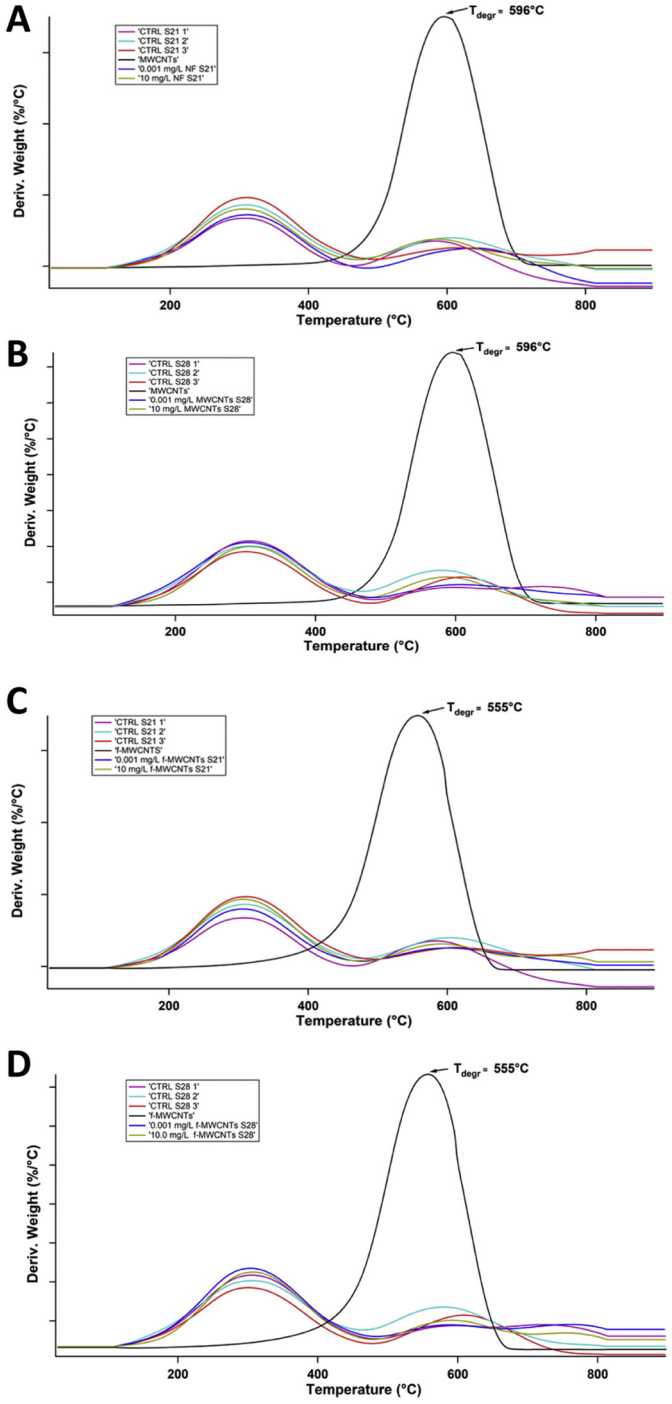


**Fig. 1.** A. DTG curves of *Diopatra neapolitana* organisms exposed to aqueous media at salinity 21 either contaminated with multiwalled carbon nanotubes (MWCNTs) dispersed at different concentrations or not contaminated. The DTG curve of multiwalled carbon nanotubes (sample MWCNTs) was used as reference; B. DTG curves of *Diopatra neapolitana* organisms exposed to aqueous media at salinity 28 either contaminated with multiwalled carbon nanotubes (MWCNTs) dispersed at different concentrations. The DTG curve of multiwalled carbon nanotubes (sample MWCNTs) was used as reference; C. DTG curves of *Diopatra neapolitana* organisms exposed to aqueous media at salinity 21 either contaminated with COOH-functionalized multiwalled carbon nanotubes (f-MWCNTs) dispersed at different concentrations or not contaminated. The DTG curve of multiwalled carbon nanotubes (sample f-MWCNTs) was used as reference; D. DTG curves of *Diopatra neapolitana* organisms exposed to aqueous media at salinity 28 either contaminated with COOH-functionalized multiwalled carbon nanotubes (f-MWCNTs) dispersed at different concentrations or not contaminated. The DTG curve of multiwalled carbon nanotubes (sample f-MWCNTs) was used as reference.

et al. (2018a, 2018b).

### 2.6. Data analysis

PERMANOVA+ add-on in PRIMER v6 was used as permutational multivariate analysis of variance of the results regarding the percentage (%) of regenerated body width, number (#) of regenerated cheatigers, PROT and GLY contents, ETS activity, LPO levels, GSH/GSSG, as well as SOD, CAT, GSTs and ATChI-ChE activities. A one-way hierarchical design was followed in this analysis. The pseudo-F *p*-values in the PERMANOVA main tests were evaluated in terms of significance. Significant differences were observed using main test and consequently pairwise comparisons were performed. Values lower than 0.05 ($p \leq 0.05$) were considered as significantly different. The null hypothesis tested was: salinity changes do not alter impacts induced by different concentrations of two MWCNTs in *D. neapolitana*. For this, three main questions were identified: i) what are the effects of tested concentrations, for each salinity level and MWCNTs type?; ii) are the effects observed dependent on the type of MWCNTs (Nf vs f), regardless the salinity and concentration levels?; iii) does salinity shifts alter organisms sensitivity to CNTs, regardless the concentration and the type of MWCNTs tested? For this we verified if: i) for each biomarker and for each salinity level, no significant differences existed between both MWCNT exposure concentrations (0.001 and 0.01 mg/L); ii) for each biomarker and for each salinity level and exposure concentration, no significant differences exist between MWCNT materials (Nf and f-MWCNTs) and iii) for each biomarker and for each MWCNTs material and exposure concentration, no significant differences exist between salinity levels (21 and 28).

## 3. Results

### 3.1. CNTs in the exposure media and organisms

#### 3.1.1. Characterization analysis in water media

As reported in the literature DLS measurements represent a well-established method for the determination of the average diameter of CNTs in aqueous dispersions (Liu et al., 2011). Here DLS analyses were repeated several times to ensure reproducible results due to the inherent heterogeneity and colloidal instability of the analyzed samples. Intensity distributions were obtained by analyzing the autocorrelation functions through the Contin algorithm which is particularly appropriate for polydisperse and multimodal systems (Varenne et al., 2016). The cumulant method was used to obtain information on the particle's average hydrodynamic radii, and on the PDI (Tardani and Mesa, 2015). The mean size (nm) and PDI of functionalized MWCNTs (f) and pristine MWCNTs (Nf) suspended particles measured in artificial seawater at different salinity levels (21 and 28) are reported in Table 1. Under salinity 21, the mean size of Nf-MWCNTs were largely higher than that of f-MWCNTs. The reliability of the mean diameter values recorded at time 28 days was compromised by the presence of microaggregates of unknown origin as evidenced by the control. Both Nf and f-MWCNTs suspended at 0.01 mg/L were found to agglomerate and remain dispersed in the medium until 28 days. The concentration of 0.001 mg/L was found to lie below the limit of instrumental detection in the adopted experimental conditions. At salinity 28, the reliability of the mean diameter values obtained at time 21 and 28 days was compromised by the presence of microaggregates of unknown origin as evidenced by the controls. Up to 14 days Nf and f MWCNTs displayed a different behavior: f-MWCNTs were found to agglomerate and remain dispersed in the medium while Nf-MWCNTs particles were not detectable by DLS analysis due to settlement and/or uptake by marine organisms. At 0.001 mg/L concentration was found to lie below the limit of instrumental detection under the adopted conditions.

**Table 1**
Dynamic Light Scattering (DLS) data of Size (nm) and Polydispersity Index (PDI) of both MWCNTs under salinity 21 and 28 (0.001 mg/L and 0.01 f-MWCNTs; 0.001 mg/L and 0.01 mg/L Nf-MWCNTs) collected at different exposure periods (T0; T7; T14; t21 and T28). I.d.: "Invalid data" (no colloidal material detected into the analyzed sample). I.d.: Invalid data (not detected colloidal material into the analyzed sample at the end of 120 acquisitions).

| Samples | Size (nm) | PDI | Size (nm) | PDI |
|---|---|---|---|---|
| | Nf-MWCNTs | | f-MWCNTs | |
| Salinity 21 | | | | |
| | T0 | | T0 | |
| 0.001 mg/L | I.d. | – | I.d. | – |
| 0.01 mg/L | 2236.0 | 1.046 | I.d. | – |
| | T7 | | T7 | |
| 0.001 mg/L | 2550.6 | 1.130 | I.d. | – |
| 0.01 mg/L | 3431.0 | 1.500 | 1963.6 | 0.840 |
| | T14 | | T14 | |
| 0.001 mg/L | I.d. | – | I.d. | – |
| 0.01 mg/L | 4191.8 | 1.918 | 2796.6 | 1.420 |
| | T21 | | T21 | |
| 0.001 mg/L | 1588.8 | 0.802 | I.d. | – |
| 0.01 mg/L | 4548.1 | 1.875 | 2912.8 | 1.874 |
| | T28 | | T28 | |
| 0.001 mg/L | I.d. | – | I.d. | – |
| 0.01 mg/L | 5588.7 | 2.123 | 7013.0 | 2.875 |
| Salinity 28 | | | | |
| | T0 | | T0 | |
| 0.001 mg/L | I.d. | – | I.d. | – |
| 0.01 mg/L | 2596.6 | 0.988 | I.d. | – |
| | T7 | | T7 | |
| 0.001 mg/L | I.d. | – | 2211.4 | 1.02 |
| 0.01 mg/L | I.d. | – | 3634.9 | 1.506 |
| | T14 | | T14 | |
| 0.001 mg/L | I.d. | – | I.d. | – |
| 0.01 mg/L | I.d. | – | 1771.2 | 0.804 |
| | T21 | | T21 | |
| 0.001 mg/L | I.d. | – | I.d. | – |
| 0.01 mg/L | 3354.7 | 1.323 | 3354.7 | 1.509 |
| | T28 | | T28 | |
| 0.001 mg/L | I.d. | – | I.d. | – |
| 0.01 mg/L | I.d. | – | 2121.3 | 0.902 |

#### 3.1.2. Characterization and bioaccumulation in the organisms

Preliminary experiments employing TGA analysis did not allow to identify the presence of MWCNTs (f and Nf) in the exposed organisms due to the overlapping of the degradation peaks recorded between 500 °C and 700 °C in the DTG curves of non-contaminated *D. neapolitana* and CNTs used as reference. The second degradation peak registered in the organisms not exposed to the contaminants strongly suggests the need for an optimization of the sample preparation for TGA analysis. To this purpose sample pre-treatment aiming to remove the material responsible for the degradation peaks overlapping that of MWCNTs in the DTG curves could represent an effective approach to raise TGA as useful technique for the detection of MWCNTs in marine organisms.

### 3.2. Physiological parameter: Regenerative capacity

The mean values for the percentage (%) of regenerated body width and the number (#) of new chaetigers in *D. neapolitana* after 11$^{th}$, 18$^{th}$ and 28$^{th}$ days of amputation are presented in Table 2 and illustrated in Fig. 2. All the results were discussed considering: i) the effects of exposure concentrations of both MWCNT materials maintained under both salinity levels (significant differences ($p \leq 0.05$) among exposure concentrations were represented with different letters: uppercase and regular letters for Nf-MWCNT at salinity 28; lowercase and regular letters for Nf-MWCNTs at salinity 21; uppercase and bold letters for f-MWCNT at salinity 28; lowercase and bold letters for f-MWCNT at salinity 21); ii) the effects of the carboxylation/functionalization of the surface of MWCNTs in organisms maintained under both salinity levels for each exposure concentration (significant differences ($p \leq 0.05$) between f-MWCNT and Nf-MWCNTs within each salinity at each exposure concentration were represented with bold hashes (#)); iii) the effects of salinity shifts in organisms exposed to both MWCNT materials in each exposure concentration (significant differences ($p \leq 0.05$) between the two salinities for each MWCNTs and exposure concentration were represented with bold asterisks (*)).

#### 3.2.1. 11$^{th}$ day

After amputation all individuals were healing the cut region, however no significant differences were observed in terms of % of regenerated body width as well as # of new chaetigers between individuals non-exposed (0.00 mg/L) and exposed to both MWCNT materials in all tested concentrations (0.001 and 0.01 mg/L) under both salinity levels (control-28 and low-21).

#### 3.2.2. 18$^{th}$ day

i) Considering the effects of exposure concentrations (0.001 vs 0.01 mg/L), for the same salinity (21 or 28) and MWCNTs (Nf or f) the results of % of regenerated body width and # of new chaetigers showed that for f-MWCNT submitted to both salinities no significant differences were observed between concentrations, while significantly lower values was detected only in individuals exposed to 0.01 mg/L Nf-MWCNTs under salinity 28 in comparison to remaining conditions.
ii) Considering the effects of MWCNTs (Nf vs f), for each concentration (0.001 or 0.01 mg/L) and each salinity (28 or 21), no significant differences were observed between organisms exposed to different MWCNTs in terms of % of regenerated body width, while regarding the # of new chaetigers, significant differences between materials were observed only in polychaetes exposed to 0.01 mg/L under salinity 28 showing a lower # of chaetigers for individuals contaminated with Nf-MWCNTs.
iii) Considering the effects of salinity (21 vs 28), for each MWCNTs (Nf or f) at each exposure concentration (0.001 or 0.01 mg/L), differences between salinities (28 and 21) were only observed at 0.01 mg/L Nf-MWCNTs with lower % of regenerated body width in individuals maintained under salinity 28 in comparison to individuals maintained under salinity 21, while no significant differences were found in terms of # of new chaetigers for both MWCNT materials (f and Nf) and for both salinities.

#### 3.2.3. 28$^{th}$ day

i) Considering the effects of exposure concentrations, the results of both % of regenerated body width and # of new chaetigers showed only significantly lower values between individuals exposed to 0.01 mg/L of Nf-MWCNTs under control salinity in comparison to the remaining concentrations, while no significant differences were observed in individuals under salinity 21 exposed to both CNTs.
ii) When comparing organisms exposed to the same salinity and exposure concentration, the % of regenerated body width as well as the # of new chaetigers did not show significant differences between individuals exposed to different MWCNT materials.
iii) For each MWCNTs (f and Nf) at each exposure concentration, differences between salinities (28 and 21) were not observed both for % of regenerated body width and # of new chaetigers.

### 3.3. Biochemical parameters

As for the physiological parameter, all the results of biochemical parameters were discussed considering: i) the effects of exposure concentrations of both MWCNT materials maintained under both salinity levels (significant differences ($p \leq 0.05$) among exposure

**Table 2**
Regeneration data (percentage (%) of body width and the number (#) of new chaetigers) for *Diopatra neapolitana*, 11, 18 and 28 days after amputation. Significant differences ($p \leq 0.05$) among exposure concentrations for each MWCNTs (f-MWCNTs and Nf-MWCNTs) and salinity (control-salinity 28 and low-salinity 21) were represented with different letters: uppercase and regular letters for Nf-MWCNT at salinity 28; lowercase and regular letters for Nf-MWCNTs at salinity 21; uppercase and bold letters for f-MWCNT at salinity 28; lowercase and bold letters for f-MWCNT at salinity 21. Significant differences ($p \leq 0.05$) between the two salinities for each MWCNTs and exposure concentration were represented with bold asterisks (*). Significant differences ($p \leq 0.05$) between f-MWCNT and Nf-MWCNTs within each salinity at each exposure concentration were represented with bold hashes (#).

| | | | 11 days | | 18 days | | 28 days | |
|---|---|---|---|---|---|---|---|---|
| | | | %body width | # chaetigers | %body width | # chaetigers | %body width | # chaetigers |
| 0.00 | Sal. 28 | f-MWCNTs | 7.67 ± 2.07$^{\mathbf{A}}$ | 0.00 ± 0.00$^{\mathbf{A}}$ | 44.64 ± 10.04$^{\mathbf{A}}$ | 21.50 ± 6.28$^{\mathbf{A}}$ | 75.79 ± 3.96$^{\mathbf{A}}$ | 30.50 ± 1.38$^{\mathbf{A}}$ |
| | | Nf-MWCNTs | 7.67 ± 2.07$^{\mathrm{A}}$ | 0.00 ± 0.00$^{\mathrm{A}}$ | 44.64 ± 10.04$^{\mathrm{A}}$ | 21.50 ± 6.28$^{\mathrm{A}}$ | 75.79 ± 3.96$^{\mathrm{A}}$ | 30.50 ± 1.38$^{\mathrm{A}}$ |
| | Sal.21 | f-MWCNTs | 9.83 ± 1.72$^{\mathbf{a}}$ | 0.00 ± 0.00$^{\mathbf{a}}$ | 45.34 ± 13.72$^{\mathbf{a}}$ | 20.00 ± 3.22$^{\mathbf{a}}$ | 74.40 ± 4.54$^{\mathbf{a}}$ | 29.83 ± 1.72$^{\mathbf{a}}$ |
| | | Nf-MWCNTs | 9.83 ± 1.72$^{\mathrm{a}}$ | 0.00 ± 0.00$^{\mathrm{a}}$ | 45.34 ± 13.72$^{\mathrm{a}}$ | 20.00 ± 3.22$^{\mathrm{a}}$ | 74.40 ± 4.54$^{\mathrm{a}}$ | 29.83 ± 1.72$^{\mathrm{a}}$ |
| 0.001 | Sal. 28 | f-MWCNTs | 7.83 ± 4.62$^{\mathbf{A}}$ | 0.00 ± 0.00$^{\mathbf{A}}$ | 43.13 ± 6.42$^{\mathbf{A}}$ | 18.83 ± 1.72$^{\mathbf{A}}$ | 72.61 ± 7.05$^{\mathbf{A}}$ | 28.17 ± 2.14$^{\mathbf{A}}$ |
| | | Nf-MWCNTs | 8.33 ± 2.73$^{\mathrm{A}}$ | 0.00 ± 0.00$^{\mathrm{A}}$ | 43.75 ± 11.39$^{\mathrm{A}}$ | 17.83 ± 3.92$^{\mathrm{A}}$ | 73.12 ± 7.74$^{\mathrm{A}}$ | 29.67 ± 1.63$^{\mathrm{A}}$ |
| | Sal.21 | f-MWCNTs | 8.00 ± 2.37$^{\mathbf{a}}$ | 0.00 ± 0.00$^{\mathbf{a}}$ | 42.36 ± 10.60$^{\mathbf{a}}$ | 18.50 ± 2.07$^{\mathbf{a}}$ | 72.37 ± 8.28$^{\mathbf{a}}$ | 29.33 ± 2.07$^{\mathbf{a}}$ |
| | | Nf-MWCNTs | 8.33 ± 1.63$^{\mathrm{a}}$ | 0.00 ± 0.00$^{\mathrm{a}}$ | 40.34 ± 3.45$^{\mathrm{a}}$ | 18.67 ± 1.03$^{\mathrm{a}}$ | 73.25 ± 6.94$^{\mathrm{a}}$ | 28.33 ± 1.97$^{\mathrm{a}}$ |
| 0.01 | Sal. 28 | f-MWCNTs | 6.50 ± 3.73$^{\mathbf{A}}$ | 0.00 ± 0.00$^{\mathbf{A}}$ | 37.87 ± 7.51$^{\mathbf{A}}$ | 18.83 ± 2.40$^{\mathbf{A\#}}$ | 70.09 ± 12.21$^{\mathbf{A}}$ | 28.67 ± 1.51$^{\mathbf{A}}$ |
| | | Nf-MWCNTs | 8.83 ± 4.53$^{\mathrm{A}}$ | 0.00 ± 0.00$^{\mathrm{A}}$ | 19.12 ± 4.83$^{\mathrm{B}*}$ | 11.17 ± 5.95$^{\mathrm{B\#}}$ | 59.41 ± 19.35$^{\mathrm{B}}$ | 26.67 ± 7.39$^{\mathrm{B}}$ |
| | Sal.21 | f-MWCNTs | 8.00 ± 2.28$^{\mathbf{a}}$ | 0.00 ± 0.00$^{\mathbf{a}}$ | 39.92 ± 6.28$^{\mathbf{a}}$ | 18.67 ± 2.16$^{\mathbf{a}}$ | 71.63 ± 9.89$^{\mathbf{a}}$ | 27.67 ± 1.37$^{\mathbf{a}}$ |
| | | Nf-MWCNTs | 7.67 ± 3.83$^{\mathrm{a}}$ | 0.00 ± 0.00$^{\mathrm{a}}$ | 39.50 ± 5.59$^{\mathrm{a}*}$ | 17.17 ± 4.26$^{\mathrm{a}}$ | 70.68 ± 5.60$^{\mathrm{a}}$ | 28.83 ± 2.14$^{\mathrm{a}}$ |

concentrations were represented with different letters in the graphics: uppercase and regular letters for Nf-MWCNT at salinity 28; lowercase and regular letters for Nf-MWCNTs at salinity 21; uppercase and bold letters for f-MWCNT at salinity 28; lowercase and bold letters for f-MWCNT at salinity 21) ii) the effects of the carboxylation/functionalization of the surface of MWCNTs in organisms maintained under both salinity levels for each exposure concentration (significant differences ($p \leq 0.05$) between f-MWCNT and Nf-MWCNTs within each salinity at each exposure concentration were represented with bold asterisks (*) in the Table 3); iii) the effects of salinity shifts in organisms exposed to both MWCNT materials in each exposure concentration (significant differences ($p \leq 0.05$) between the two salinities for each MWCNTs and exposure concentration were represented with bold asterisks (*) in the graphics).

### 3.3.1. Energy reserves and metabolic capacity

#### 3.3.1.1. Protein (PROT) content.

i) Considering the effects of exposure concentrations, results of PROT content in *D. neapolitana* exposed to 0.01 mg/L of Nf-MWCNT under salinity 28 showed significantly higher PROT content in comparison to the remaining concentrations, while in individuals exposed to f-MWCNTs under both salinities no significant differences were observed among exposure concentrations (Fig. 3A).

ii) When comparing *D. neapolitana* exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed only in polychaetes exposed to 0.01 mg/L under salinity 28 showing an increase of the content for individuals contaminated with Nf-MWCNTs (Table 3).

iii) Significant differences between salinities (28 and 21) were observed in PROT content when organisms were exposed to 0.01 mg/L of Nf-MWCNTs, showing higher content in individuals maintained at salinity control in comparison to low salinity (Fig. 3A).

#### 3.3.1.2. Glycogen (GLY) content.

i) Along the increasing Nf-MWCNTs exposure concentrations, all the exposed polychaetes maintained at salinity 21 decreased significantly their GLY content in comparison to non-exposed ones (Fig. 2B), while under salinity 28 no significant differences were observed between contaminated and non-contaminated organisms. Opposite results were observed in organisms submitted to f-MWCNTs, showing a significantly decrease on the GLY content in exposed individuals under salinity 28 in comparison to the control, while no significant differences between different concentrations and control were observed when *D. neapolitana* were submitted to low salinity 21 (Fig. 3B).

ii) When comparing *D. neapolitana* exposed to the same salinity and

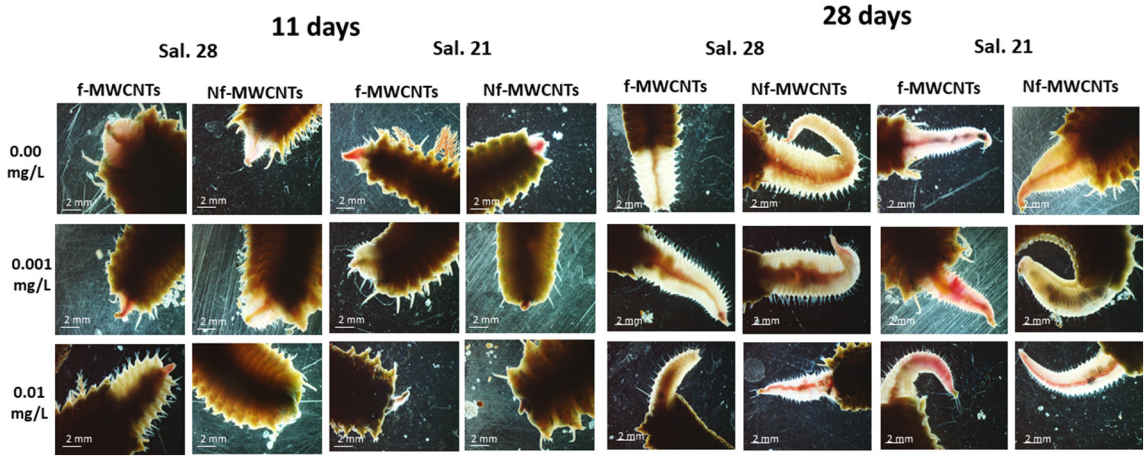


**Fig. 2.** Regenerative capacity of *Diopatra neapolitana* at 11$^{th}$ and 28$^{th}$ days after amputation, exposed to different MWCNT materials (f and Nf) and concentrations (0.00; 0.001 and 0.01 mg/L) under different salinity levels (control-28 and low-21).

**Table 3**
Effect on oxidative stress biomarkers (PROT, GLY, ETS, LPO, GSH/GSSG SOD, CAT, GSTs, ATChI-ChE) in *Diopatra neapolitana* by f-MWCNTs and Nf-MWCNTs at each of the tested concentrations (control-0.00, 0.001, 0.01 mg/L) under control-salinity 28 and low-salinity 21. Significant differences ($p \leq 0.05$) between f-MWCNT and Nf-MWCNTs within each salinity at each exposure concentration were represented with asterisks.

| | | | PROT | GLY | ETS | LPO | GSH/GSSG | SOD | CAT | GSTs | ATChI-ChE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.00 | Sal.28 | f-MWCNTs | 39.45 ± 9.03 | 1.51 ± 0.21 | 23.47 ± 2.29 | 12.83 ± 0.94 | 6.83 ± 0.45 | 0.83 ± 0.21 | 39.68 ± 3.10 | 0.34 ± 0.04 | 0.98 ± 0.14 |
| | | Nf-MWCNTs | 39.45 ± 9.03 | 1.53 ± 0.19 | 23.47 ± 2.29 | 12.83 ± 0.94 | 6.83 ± 0.45 | 0.83 ± 0.21 | 39.68 ± 3.10 | 0.34 ± 0.04 | 0.98 ± 0.14 |
| | Sal.21 | f-MWCNTs | 37.56 ± 5.30 | 1.51 ± 0.21 | 23.63 ± 2.45 | 13.80 ± 0.76 | 6.91 ± 0.30 | 1.02 ± 0.46 | 39.10 ± 1.38 | 0.32 ± 0.04 | 0.97 ± 0.15 |
| | | Nf-MWCNTs | 37.56 ± 5.30 | 1.53 ± 0.19 | 23.63 ± 2.45 | 13.80 ± 0.76 | 6.91 ± 0.30 | 1.02 ± 0.46 | 39.10 ± 1.38 | 0.32 ± 0.04 | 0.97 ± 0.15 |
| 0.001 | Sal.28 | f-MWCNTs | 36.70 ± 7.00 | 1.23 ± 0.25* | 27.08 ± 4.10 | 15.23 ± 1.37 | 6.05 ± 0.41 | 2.74 ± 1.24* | 37.88 ± 4.17 | 0.32 ± 0.04* | 0.88 ± 0.10 |
| | | Nf-MWCNTs | 36.26 ± 3.88 | 1.52 ± 0.08 | 25.52 ± 3.00 | 13.52 ± 2.14 | 6.71 ± 0.75 | 1.05 ± 0.47 | 38.63 ± 1.57 | 0.27 ± 0.03 | 0.91 ± 0.14 |
| | Sal.21 | f-MWCNTs | 34.85 ± 3.86 | 1.40 ± 0.14 | 26.03 ± 2.53 | 15.14 ± 1.35 | 6.01 ± 0.56 | 0.87 ± 0.41 | 38.59 ± 0.82 | 0.24 ± 0.04 | 0.85 ± 0.12 |
| | | Nf-MWCNTs | 37.35 ± 5.17 | 1.21 ± 0.16 | 25.57 ± 3.83 | 14.93 ± 2.51 | 6.45 ± 0.66 | 1.25 ± 0.64 | 38.99 ± 1.81 | 0.26 ± 0.03 | 0.87 ± 0.16 |
| 0.01 | Sal.28 | f-MWCNTs | 36.24 ± 8.02* | 1.19 ± 0.14* | 29.52 ± 2.06* | 19.11 ± 2.97* | 5.26 ± 0.60* | 2.86 ± 0.85* | 39.55 ± 3.71* | 0.26 ± 0.03* | 0.79 ± 0.16* |
| | | Nf-MWCNTs | 81.33 ± 10.09 | 1.45 ± 0.07 | 20.29 ± 1.83 | 29.59 ± 2.88 | 2.16 ± 0.27 | 1.09 ± 0.11 | 38.63 ± 5.15 | 0.86 ± 0.04 | 1.28 ± 0.71 |
| | Sal.21 | f-MWCNTs | 35.91 ± 3.36 | 1.25 ± 0.22 | 28.31 ± 3.61 | 16.81 ± 3.04 | 6.50 ± 0.60 | 2.41 ± 0.63* | 38.98 ± 1.28 | 0.27 ± 0.02 | 0.88 ± 0.14 |
| | | Nf-MWCNTs | 35.31 ± 6.67 | 1.26 ± 0.18 | 25.96 ± 3.83 | 16.15 ± 2.37 | 6.88 ± 0.69 | 1.10 ± 0.51 | 38.99 ± 1.38 | 0.30 ± 0.03 | 0.88 ± 0.13 |

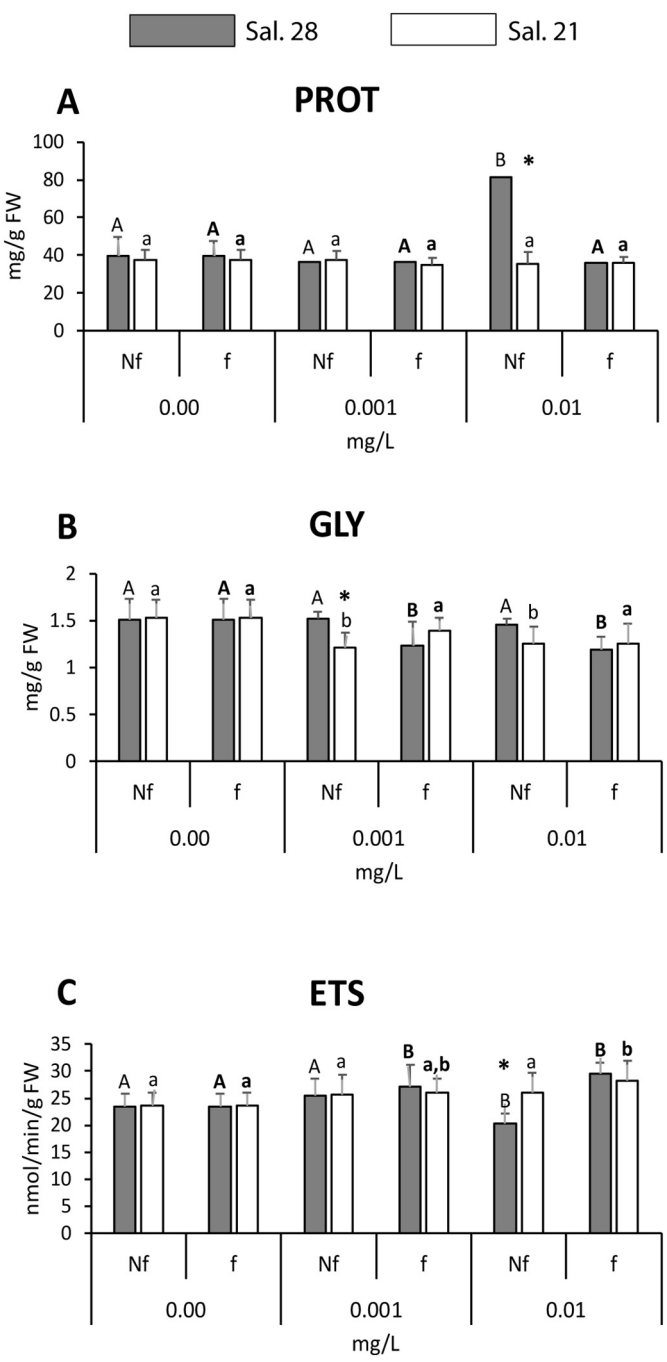


**Fig. 3.** A. Protein (PROT) content; B. Glycogen (GLY) content; C. Electron transport system (ETS) activity (mean + standard deviation) in *Diopatra neapolitana* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.10 and 1.00 mg/L) under different salinities (control-28 and low-21).

exposure concentration, significant differences between MWCNT materials (f and Nf) were observed in organisms exposed to both concentrations at salinity 28, showing decrease of the content in individuals contaminated with f-MWCNTs (Table 3).

iii) For each of the MWCNT (f and Nf) and exposure concentration, differences between salinities (28 and 21) were observed only at lowest exposure concentration (0.001 mg/L) for specimens under Nf-MWCNTs, with significantly higher GLY content in organisms maintained to salinity 28 in comparison to the specimens under salinity 21 (Fig. 3B).

*3.3.1.3. Electron transport system (ETS) activity.*

i) At salinity 28 *D. neapolitana* presented a significant decrease of ETS activity only at 0.01 mg/L Nf-MWCNTs in comparison to the remaining conditions, while at salinity 21 no significant differences were observed among concentrations. Regarding individuals exposed to f-MWCNTs, significantly higher ETS activity was observed in contaminated individuals under both salinity levels compared to control (Fig. 3C).
ii) When comparing specimens exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed only in polychaetes exposed to 0.01 mg/L under salinity 28 showing lower activity for individuals contaminated with Nf-MWCNTs in comparison to individuals exposed to f-MWCNTs (Table 3).
iii) For each MWCNT (f and Nf) and exposure concentration, differences between salinities (28 and 21) were only observed at 0.01 mg/L Nf-MWCNTs, with lower ETS activity in individuals maintained at salinity 28 in comparison to organisms under salinity 21 (Fig. 3C).

*3.3.2. Indicators of oxidative stress*
*3.3.2.1. Lipid peroxidation (LPO) level.*

i) Under salinity 28 the level of LPO in polychaetes exposed to Nf-MWCNTs increased at the highest exposure concentration (0.01 mg/L) with significant differences in comparison to the other treatments, while in organisms under salinity 21 the LPO at 0.001 and 0.01 mg/L was significantly higher than levels observed in non-exposed organisms, and no significant differences were observed between individuals exposed to these two concentrations (Fig. 4A). Considering the organisms exposed to f-MWCNTs and maintained at salinity control, the level of LPO increased with the increasing of exposure concentrations with significant differences among all concentrations, while under low salinity significantly lower LPO level was observed in all contaminated *D. neapolitana* in comparison to control organisms.
ii) When comparing individuals exposed to different MWCNTs at the same salinity and exposure concentration, significant differences between materials were observed only in polychaetes exposed to 0.01 mg/L under salinity 28 with higher values in individuals contaminated with Nf-MWCNTs in comparison to organisms contaminated with f-MWCNTs (Table 3).
iii) For each of the MWCNT (f and Nf) and exposure concentration, differences between salinities (28 and 21) were observed only at the highest exposure concentration for specimens under Nf-MWCNTs, with significantly higher LPO level in organisms maintained to control salinity 28 in comparison to polychaetes under salinity 21 (Fig. 4A).

*3.3.2.2. Reduced (GSH) and oxidized (GSSG) glutathione ratio.*

i) Significantly lower ratio between GSH and GSSG was observed in organisms contaminated with 0.01 mg/L Nf-MWCNTs under salinity 28 in comparison to the remaining concentrations, while no significant differences were observed in specimens maintained at salinity 21. Under salinity 28, GSH/GSSG content in *D. neapolitana* exposed to f-MWCNTs decreased with the increasing of exposure concentrations with significant differences among all treatments. A similar trend was also observed for individuals submitted to salinity 21, however no significant differences were found between contaminated organisms (Fig. 4B).
ii) When comparing organisms exposed to the same salinity and exposure concentration, significant differences between MWCNT materials (f and Nf) were observed only in *D. neapolitana* exposed to 0.01 mg/L at salinity 28, showing the lowest ratio in individuals contaminated with Nf-MWCNTs (Table 3).
iii) For each of the MWCNT (f and Nf) and exposure concentration, differences between salinities (28 and 21) were observed in polychaetes exposed to the highest concentration of MWCNTs (both f and Nf), with lower ratio in individuals maintained at control salinity compared to individuals under salinity 21 (Fig. 4B).

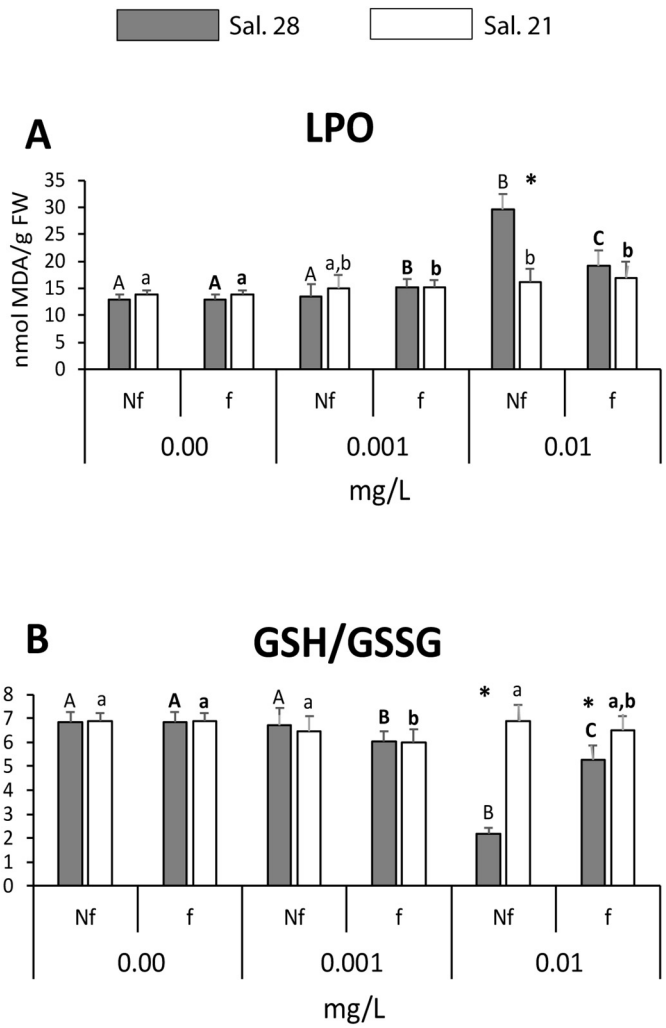


**Fig. 4.** A. Lipid peroxidation (LPO) levels; B. GSH/GSSG (mean + standard deviation) in *Diopatra neapolitana* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.10 and 1.00 mg/L) under different salinities (control-28 and low-21).

*3.3.2.3. Superoxide dismutase (SOD) activity.*

i) Considering the effects of exposure concentrations, results of SOD activity in *D. neapolitana* showed that for Nf-MWCNTs under both salinities (28 and 21), no significant differences were observed among all conditions. In polychaetes exposed to f-MWCNTs the SOD activity significantly increased with the increasing exposure concentrations under salinity 28, while under salinity 21 the activity of this enzyme significantly increased at the highest exposure concentration (0.01 mg/L) in comparison to the other treatments (Fig. 5A).
ii) Comparing organisms under the same salinity and exposure concentration, significantly higher SOD activity at 0.001 mg/L was observed in polychaetes exposed to f-MWCNTs compared to Nf-MWCNTs under salinity control, while at 0.01 mg/L significant differences between materials were found in organisms maintained

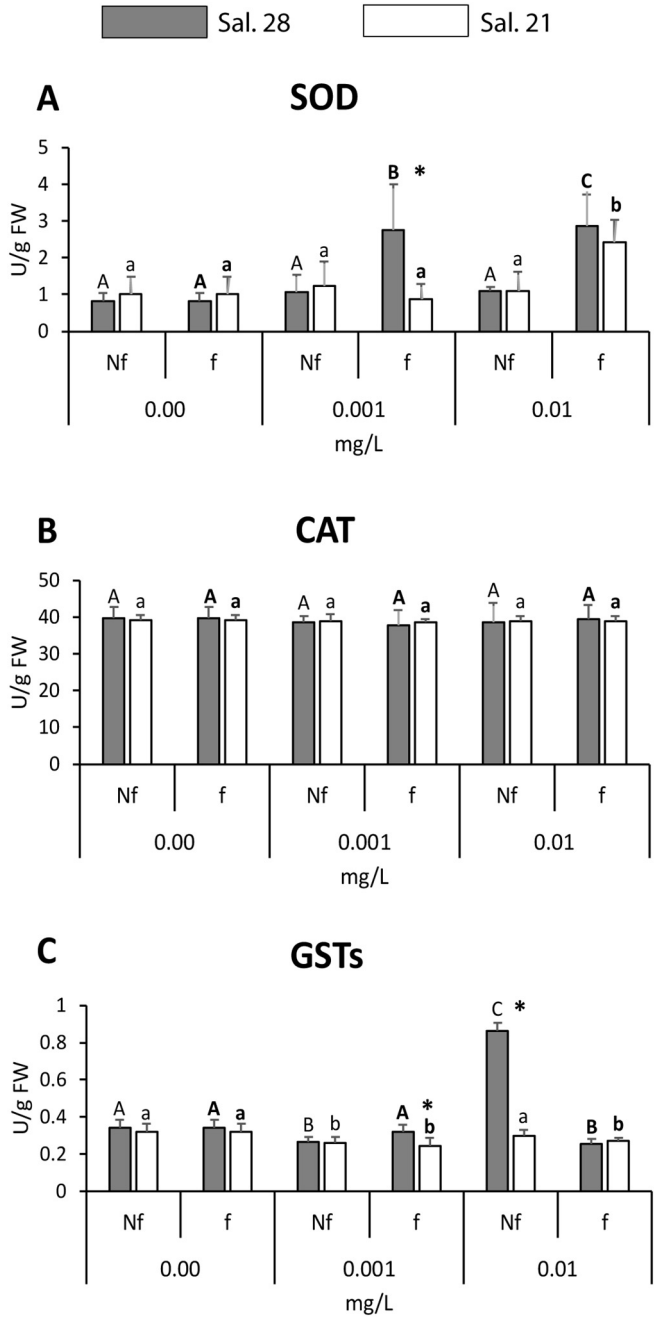


**Fig. 5.** A. Superoxide dismutase (SOD) activity; B. Catalase (CAT) activity; **C.** Glutathione S-transferases (GSTs) activity (mean + standard deviation) in *Diopatra neapolitana* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.10 and 1.00 mg/L) under different salinities (control-28 and low-21).

under both salinities (21 and 28), showing higher activity in individuals contaminated with f-MWCNTs (Table 3).

iii) For each of the MWCNT (f and Nf) and exposure concentration, differences between salinities (28 and 21) were observed only at the lowest exposure concentration (0.001 mg/L) for specimens under f-MWCNTs, with significantly higher SOD activity in organisms maintained to control salinity 28 in comparison to organisms under salinity 21 (Fig. 5A).

*3.3.2.4. Catalase (CAT) activity.*

i) Considering the effects of exposure concentrations, the results of CAT activity in organisms exposed to both MWCNT materials and under both salinity levels did not evidence any significant differences between concentrations (Fig. 5B).

ii) When comparing organisms exposed to the same salinity and exposure concentration, significant differences between MWCNT materials (f and Nf) were observed only in *D. neapolitana* exposed to 0.01 mg/L at salinity 28, showing higher activity in individuals contaminated with f-MWCNTs (Table 3).

iii) For each of the MWCNTs (f and Nf) and exposure concentration, no significant differences in terms of CAT activity were observed between salinities (28 and 21) (Fig. 5B).

*3.3.2.5. Glutathione S-transferases (GSTs) activity.*

i) Polychaetes maintained at salinity 28 decreased significantly the activity of GSTs when exposed to 0.001 mg/L Nf-MWCNTs, but at the highest exposure concentration (0.01 mg/L) the enzyme activity significantly increased to values higher than control levels. Under salinity 21 significantly lower GSTs activity was observed in contaminated organisms with 0.001 mg/L in comparison to control individuals, however at 0.01 mg/L the activity reached again the same value of non-contaminated individual without significant differences. When organisms were exposed to f-MWCNTs under salinity 28 the activity significantly decreased only at the highest exposure concentration (0.01 mg/L) in comparison to the other treatments, while under salinity 21 the GSTs activity decreased in all contaminated organisms in comparison to non-contaminated ones (Fig. 5C).

ii) When comparing organisms exposed to the same salinity and exposure concentration, significant differences between polychaetes exposed to different MWCNTs were observed at 0.001 mg/L, with lower activity in *D. neapolitana* exposed to Nf-MWCNTs under salinity 28 compared to individuals exposed to f-MWCNTs. Significant differences between materials were also observed in individuals exposed to 0.01 mg/L maintained under salinity 28, showing an opposite response with higher activity in individuals contaminated with Nf-MWCNTs in comparison to f-MWCNTs (Table 3).

iii) For each of the MWCNTs (f and Nf) and exposure concentration, slight differences between salinities (28 and 21) were observed between organisms exposed to 0.001 mg/L f-MWCNTs, showing higher GSTs activity under salinity 28 compared to individuals under salinity 21. Significant differences between salinities were also recorded in individuals submitted to 0.01 mg/L Nf-MWCNTs, with higher enzyme activity in individuals under salinity 28 in comparison to low salinity (Fig. 5C).

*3.3.3. Neurotoxicity*

*3.3.3.1. Acetylcholinesterase (AChI-ChE) activity.*

i) At salinity 28 *D. neapolitana* presented a significant increase of AChI-ChE activity only at 0.01 mg/L Nf-MWCNTs, while under salinity 21 no significant differences were observed between concentrations. Polychaetes exposed to f-MWCNTs under both salinities showed no significant differences in the AChI-ChE activity between different treatments (Fig. 6).

ii) When comparing organisms exposed to the same salinity and exposure concentration but different MWCNTs significantly higher neuro-enzyme activity was only recorded in organisms contaminated with 0.01 mg/L Nf-MWCNTs at salinity 28 in comparison to f-MWCNTs (Table 3).

iii) For each of the MWCNTs (f and Nf) and exposure concentration, differences between salinities (28 and 21) were observed only between organisms exposed to 0.01 mg/L Nf-MWCNTs, showing higher AChI-ChE activity under salinity 28 compared to individuals under salinity 21 (Fig. 6).

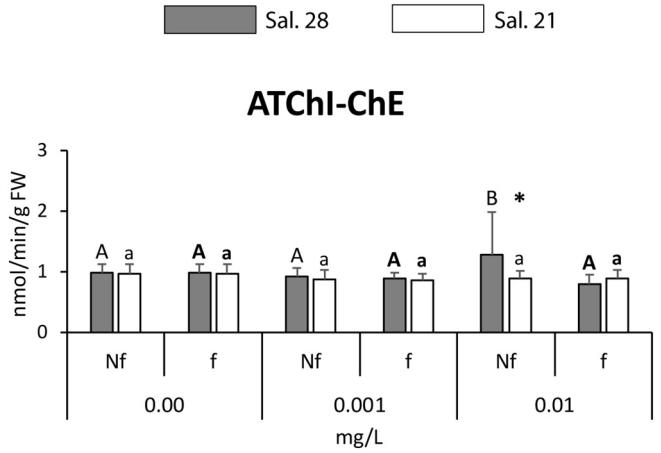


**Fig. 6.** Acetylcholinesterase (ATChI-ChE) activity in *Diopatra neapolitana* exposed to different MWCNT materials (Nf-MWCNTs and f-MWCNTs) both at different concentrations (0.00; 0.10 and 1.00 mg/L) under different salinities (control-28 and low-21).

## 4. Discussion

The two main questions that have been investigated in the present study were I) toxic impacts observed in *D. neapolitana* can be influenced by concentrations and the surface chemistry alteration/functionalization of CNTs; II) the sensitivity of the polychaetes and/or the toxicity of the CNTs can be changed by salinity.

I) In the present study, the toxic impact of surface chemistry functionalization of CNTs on this species was observed in terms of physiological responses, energy reserves and metabolic capacity as well as in their oxidative and neuro status. In detail, our results demonstrated that Nf-MWCNTs under control salinity have a negative effect on the regenerative capacity of *D. neapolitana* at the highest exposure concentration showing lower percentage of body width as well as the number of new chaetigers compared to the other conditions after 18th and 28th days exposure. Similar responses were also demonstrated by De Marchi et al. (2017d) exposing the same species to the same CNTs. Other studies also showed that CNTs can induce alterations in physiological functions in different invertebrate species (Moschino et al., 2014; Mwangi et al., 2012). For example, Moschino et al. (2014) demonstrated sub-lethal effects at the digestion level in the polychaete *Hediste diversicolor* exposed to three single walled carbon nanohorns (SWCNHs) and Mwangi et al. (2012) showed that both MWCNTs and SWCNTs significantly reduced the survival and growth of an amphipod (*Hyalella azteca*), a midge (*Chironomus dilutus*), an oligochaete (*Lumbriculus variegatus*), and a mussel (*Villosa iris*). Generally the toxic effects of different pollutants are usually associated with changes in the energy distribution in invertebrates due to increased energy expenses associated with detoxification processes (Bednarska et al., 2013). However, in the present study, *D. neapolitana* presented a decrease of electron transport system (ETS) activity and an increase of glycogen (GLY) and protein (PROT) contents when exposed to the highest Nf-MWCNTs concentration and maintained at control salinity. Different studies already demonstrated that when organisms are submitted to different pollutants, oxidative stress may occur as a consequence of reactive oxygen species (ROS) generation, causing lipid peroxidation (LPO) of the mitochondria membranes, thus impairing the function of ETS activity (Choi et al., 2001; Bielen et al., 2016). This hypothesis could explain partially why in the presence of high contaminant level, the organisms showed a decrease of metabolic rate preventing the consumption of energy reserves. However, an opposite behavior was observed in individuals exposed to f-MWCNTs under control salinity, showing no differences in terms of regenerative capacity but a decrease of energy reserves (especially GLY content) with a consequent increase of metabolic capacity (ETS activity). It has been already demostarted that behavioral/physiological responses to stress may increase energy demand (Sokolova et al., 2012), which could indicate that polychaetas under this condition were using their energy reserves to regenerate their body fighting against high CNTs concentration. In fact, PROT as well as GLY are used by the organisms as main energy reserves (Beninger and Lucas, 1984) to preserve cellular damage when exposed to pollutants (Klaper et al., 2010). Moreover, the increase of ETS could be due to the activation of defense mechanisms, such as the increase on superoxide dismutase (SOD) activity, as demonstrated under this exposure condition in contaminated organisms with f-MWCNTs. Similar results were also obtained by Bertrand et al. (2016) which exposing the bivalve *Scrobicularia plana* to silver (Ag) NMs, observed an increase of ETS activity indicating impairment of metabolic activity in clams that suffered from LPO of their cellular membranes and activation of antioxidant enzymes. The controversial behavior of energy reserves and metabolic activity observed in the present study could be attributed to the surface functionalization of the CNTs. While raw CNTs do not readily cross biological barriers due to low dispersibility and low resident time in the water column, water dispersible MWCNTs (as for COOH-MWCNTs used in the present study), due to the presence of higher amorphous carbon fragments in comparison to pristine MWCNTs, induced higher levels of toxicity to biological systems (Arndt et al., 2013) causing higher cellular damage with the activation of antioxidant mechanisms (Freixa et al., 2018). This hypothesis was confirmed in the present results due to a greater antioxidant enzymes activity such as SOD in organisms exposed to f-MWCNTs compared to Nf-MWCNTs.

Interactions of CNTs with organisms can be external, as attachment of the NMs onto the skin or exoskeleton, or internal, via food intake, or both (Mesarič et al., 2015). All of these interactions can cause different physiological disturbances, as also demonstrated in the present study, as well as the generation of oxidative stress, with direct damage of the lipid membranes due to the high affinity of CNTs for lipid membranes and a consequent activation of antioxidant enzymes (Mesarič et al., 2015). These responses were already demonstrated when polychaetes were exposed to different carbon NMs (De Marchi et al., 2017c, 2017d; Monserrat et al., 2017). However, successful CNTs uptake in the exposed organisms are important prerequisites for bioaccumulation in the body and consequent cellular damage which are directly related to the characteristics of the CNTs such as heterogeneous purity, length, type of functionalization (Costa et al., 2016). Looking to the results of the present study, while in the organisms exposed to Nf-MWCNTs under salinity control the LPO increased only at the highest exposure concentration, in polychaetes exposed to f-MWCNTs the damage of the lipid membranes was also observed at the lowest exposure concentration, assuming that these different responses were directly related to the availability of the CNT materials. While Nf-MWCNTs, due to their poor suspendability as demonstrated by DLS analysis, could be less available for the organisms, f-MWCNTs were more dispersible in the water column probably increasing their mobility and thus may intensify the risk of exposure and toxicity and possible uptake (Jackson et al., 2013).

The generation of LPO is known to be responsible for the activation/inactivation of the antioxidant defense system (Lapresta-Fernández et al., 2012). Reduced glutathione (GSH)/oxidized glutathione (GSSG), considered as a regulatory molecule and sensor of the redox state of cells (Mocan et al., 2010), and antioxidant enzymes play an important role in organisms defense system against oxidative damage (Ighodaro and Akinloye, 2017). In the present study, the polychaetes exposed to f-MWCNTs under salinity control showed dose-dependent increased of the LPO with a consequence dose-dependent decrease of GSH/GSSG as well as increase of SOD activity. This result suggested a compensatory response of cellular defense systems against cellular damage, while in organisms exposed to Nf-MWCNTs under control salinity, the GSH/GSSG decreased at the highest exposure concentration but the SOD activity did not increase under this condition. This behavior may be due

to an excessive ROS production, especially under the highest exposure concentration, leading to oxidative damage and a loss of compensatory mechanisms as a consequence of insufficient antioxidant mechanisms (Fukai and Ushio-Fukai, 2011; Walters et al., 2016) which may contribute to higher LPO levels recorded at this condition.

As multicomponent enzymes involved in the detoxification of different xenobiotics, glutathione S-transferases (GSTs) play important roles in protecting tissues from oxidative stress (Fournier et al., 1992) and they have been already used as biomarkers of cellular damage as these enzymes exhibit many of the required characteristics, i.e. specific localization, high cytosolic concentration and relatively short half-life (Pérez et al., 2004). In previously published studies GSTs showed different mechanisms of action when exposed to different NMs, assuming that GSTs activity may be either increased or decreased due to production of lipid hydroperoxides (Kos et al., 2017) and also the type of NMs (Lehman et al., 2011). For example, Canesi et al. (2010) exposing the *M. galloprovincialis* to different CNMs (nano carbon black-nNCB, C60 fullerene), reported that all CNMs induced changes in GSTs activities, with contrasting trends, depending on NMs type and solubility. The results of the present study are in line with such findings, showing a decreased GSTs activities when organisms were exposed to Nf-MWCNTs (insoluble) and increased activities in organisms exposed to f-MWCNTs (soluble), both under control salinity.

Neurotoxicity was also investigated in the present study, evaluating the activity of acetylcholinesterase, ATChI-ChE, which are specific esterases that mainly hydrolyse choline-based esters, several of which are used as neurotransmitters (Mennillo et al., 2017; Augustinsson, 1971). Although in recent years the number of studies that investigated the interactions between ChE and NMs have been increasing, generally demonstrating an inhibition of their activity in invertebrates as a consequence of CNTs exposure (De Marchi et al., 2017a, 2017b, 2017c; Monserrat et al., 2017; De Marchi et al., 2018a, 2018b), opposite results were observed in the present study, showing no inhibition of ATChI-ChE activity in exposed organisms under both materials and both salinities. Such result may be related to the fact that organisms try to reduce neurotransmitter excess in the synaptic clefts, which was already showed in the bivalve *Perna indica* exposed to arsenic (As) (Rajkumar, 2013).

II) Alteration induced by salinity shifts modifying the sensitivity of the polychaetes and the toxicity of the CNTs was also observed in the present study. It was already demonstrated that organisms exposed to salinity stress must increase their energy expenditure to successfully acclimate to the stressor and ensure cellular protection (Rivera-ingraham, 2017). When organisms are exposed to low salinity level initiate a series of mechanisms (energetically costly) that allow them to hyper-regulate (i.e. to maintain their extracellular fluids at a higher osmolality than that of their surrounding medium) and this osmoregulation is considered to be an energetically costly process (Rivera-ingraham, 2017). This hypothesis supported our results, showing that when the polychaetes were exposed to salinity 21 under both MWCNT materials, there was an increase of the energy expenditure (showed by decrease of the GLY) and an increase of metabolic activity (expressed by an increase of ETS activity) demonstrating that the alteration induced by salinity shifts modifying the sensitivity of the polychaetes to the CNTs, but did not modified the toxicity of the NMs, as for both exposure conditions (f and Nf) it was possible to observe the same trend in terms of energy reserves consumption and metabolic activity in the exposed organisms.

Mitochondria, as the main energy producers in eukaryotic cells, play a central role in acclimation processes. However, they also represent the main source of reactive oxygen and nitrogen species (ROS/RNS), although the relationship between mitochondrial respiration and ROS/RNS formation is not fully understood. ROS/RNS can potentially lead to the LPO (such as those composing cellular membranes), as well as damaging other cellular molecules; ROS/RNS potentially have negative consequences for acclimation to hyper- and hypo-osmotic conditions. However, the lipid electrophiles resulting from such processes can have, along with ROS/RNS themselves, a role in the activation of cellular defences (Rivera-ingraham, 2017; Sokolova, 2018). In the present study, organisms exposed at both f-MWCNTs and Nf-MWCNTs under low salinity presented an increase of LPO and activation of antioxidant enzymes in terms of increase of SOD activity as well as decrease of GSH/GSSG and decrease of GSTs especially at the highest exposure concentration, demonstrating that the alteration induced by salinity shifts on the sensitivity of the polychaetes to low salinity caused major toxicity in comparison to the chemical behavior of both MWCNTs under this condition. In fact, despite estuarine invertebrates are often exposed to short-term (tidal) and long-term (rain periods) changes in salinity, the increased stress may lead to physiological and morphological abnormalities when exposed to low salinity (Verdelhos et al., 2015).

Nevertheless, in general, in the present study both Nf-MWCNTs and f-MWCNTs under salinity 28 generated greater alterations on energy reserves and metabolic activity, oxidative stress biomarker responses and antioxidant enzymes activities compared to individuals maintained under salinity 21, assuming that exposed polychaetes tend to be more sensitive to the alteration induced by salinity variations on the chemical behavior of both MWCNTs in comparison to salt stress. It has been already demonstrated from the literature that higher salinity causes the formation of large-size aggregates (Hu et al., 2017) which can alter their biological effects by affecting ion release from the surface and their reactive surface area, affecting the mode of cellular uptake of NMs together with subsequent biological responses in the organisms (Hotze et al., 2010). Ward and Kach (2009) revealed that the larger aggregates can considerably increase the uptake of polystyrene NMs by suspension filter-feeding bivalves (the mussels *M. edulis* and oysters *Crassostrea virginica*), which were either dispersed or embedded within aggregates, showing that both these species more efficiently captured and ingested NMs that were incorporated into aggregates compared to those freely suspended. These findings are in agreement with our results, showing major toxic impacts in organisms exposed to higher salinity 28.

## 5. Conclusions

The results of the present study demonstrated clearly that both CNTs generated toxic impacts in terms of energy reserves and metabolic capacity as well as oxidative and neuro status. However, when comparing f-MWCNTs and Nf-MWCNTs, greater toxic impacts in the polychaetes *D. neapolitana* were observed by functionalized CNTs due to availability of the CNT materials. While Nf-MWCNTs, due to their insolubility, may be less available for the organisms, f-MWCNTs were more dispersible in the water column probably increasing their mobility and thus increasing the risk of exposure, possible uptake and toxicity, leading to a much higher cellular damage concluding that nanomaterial toxicity can be attributed to core structure and surface functionalization. Moreover, when evaluating if the alteration induced by salinity shifts could modify the sensitivity of the polychaetes and/or the toxicity of the CNTs, the present findings demonstrated that Nf-MWCNTs and f-MWCNTs under salinity 28 generated greater toxic impacts in the polychaetes compared to individuals maintained under salinity 21, assuming that the alteration induced by salinity shifts on the chemical behavior of both MWCNTs and consequent fate in exposed polychaetes caused major toxicity in comparison to the sensitivity of the organisms to low salinity. Thus, nanomaterials toxicity was not only attributed to core structure and surface functionalization, but also to the physico-chemical parameters of the media which alter the behavior of the CNTs and consequently the toxicity in the exposed organisms. Considering that CNTs are one of the most promising classes of new materials to emerge from nanotechnology to date, the results of these studies provided essential information about the behavior of commercially important CNTs in complex physiological environment giving a scientifically grounded knowledge base for the risk assessment of these materials. Moreover, the complex interactions between climate change and pollutants may

be particularly problematic for species living at the edge of their physiological tolerance range where acclimation capacity may be limited. On this topic, understanding how pollutants behave once reaching the environment, and how different climate related factors (e.g. salinity shifts) may influence their fate, transport and toxicity, will be of major relevance to predict interactions between climate change and contaminant exposures.

Based on the results here presented, data obtained highlight the need to develop standard protocols for CNTs toxicological testing to characterize the behavior and fate of these materials in different compartments of the aquatic environment, and identify exposure conditions following environmental relevant concentrations. Also, it was point out the importance to use a broad range of biomarkers to evaluate the possible toxic effects of these new emerging pollutants. Moreover, this study improved the understanding of biological responses of polychaetes exposed to combined CNTs and predicted climate change scenarios.

## Conflict of interest

The Authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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